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# 1 Summary

Prostate cancer is one of the leading causes of cancer related deaths in males worldwide. Therefore there is a vast interest in trying to understand the exact reasons for this disease. In the last years several studies were able to expose more and more of the molecular mechanisms underlying prostate cancer. However, there are still a lot of areas which remain a black box. One of these is the correlation of inflammation and prostate tumor development. While there are several hints and hypotheses regarding this correlation, a lot of additional work is needed to finally be able to decipher this mechanism. This study is one further step in this direction.

We used a mouse model expressing a constitutively active IKK2 in the prostate. IKK2 is a key mediator in the NF $\kappa$ B pathway and its constitutive activation leads to a permanent inflammatory signaling. Interestingly inflammatory signaling alone is not enough for the transformation of the prostate. However, in combination with the heterozygous knockout of the tumor suppressor PTEN inflammatory signaling does lead to tissue transformation and an increased proliferation.

Real time RT PCR experiments revealed several up- and downregulated genes. Especially the expression of specific cytokines and chemokines seemed to be severely altered in the double transgenic mouse model. We reasoned that these chemokines were responsible for the infiltration of immune cells into the prostatic tissue. We detected these immune cells by additional immunohistochemical stainings.

Furthermore, we discovered the downregulation of smooth muscle cell markers and a resultant loss of smooth muscle throughout the epithelium. This is a known finding in prostate cancer and is usually correlated with tumor invasion, which, however, did not develop in our model. Therefore the loss of smooth muscle apparently is not enough to promote tumor invasiveness.

All in all, this study gives some new insights into how inflammation could promote prostate tumor formation when combined with a tissue with a neoplastic transformation.

## **2 Zusammenfassung**

Prostatakrebs ist eine der führenden Todesursachen von an Krebs erkrankten Männern weltweit. Deshalb herrscht reges Interesse daran, die exakten Hintergründe dieser Krankheit zu erforschen und verstehen. Obwohl in den vergangenen Jahren verschiedene Studien immer mehr Details über die molekularen Mechanismen ans Licht bringen konnten, verbleiben viele Vorgänge unverstanden. Einer dieser Vorgänge ist der Zusammenhang zwischen Entzündung und der Entwicklung von Prostatakrebs. Mit dieser Studie wollen wir dazu beitragen, die unzähligen Vermutungen und Hypothesen dieser Korrelation zu bestätigen oder zu widerlegen.

In diesem Projekt haben wir dazu mit einem Mausmodell gearbeitet, das ein konstitutiv aktivierte IKK2 im Prostatagewebe aufweist. IKK2 spielt im Signalweg von NFκB eine zentrale Rolle und seine konstitutive Aktivierung führt zu einer andauernden Entzündungsreaktion. Diese Entzündungsreaktion alleine reicht allerdings nicht aus, um zu einer Transformation der Prostata zu führen. Erst in Kombination mit einem heterozygoten knock out des Tumor Suppressors PTEN kommt es zu einer Transformation der Prostata und zu einer erhöhten Proliferationsrate.

Real time RT PCR Experimente enthüllten einige hinauf, bzw. hinunter regulierte Gene im doppelt transgenen Mausmodell. Bei vielen davon handelte es sich um Cytokine und Chemokine. Wir schlussfolgerten, dass diese Chemokine für die Infiltration des Gewebes durch Immunzellen, die wir mit mittels immunohistochemischen Stainings sichtbar machen konnten, verantwortlich zeichneten.



Desweiteren entdeckten wir eine drastische Hinunterregulierung von sogenannten „smooth muscle cell markers“ und eine daraus resultierende Abnahme von glatter Muskulatur im Epithel. Diese Reduktion von glatter Muskulatur ist ein bekanntes Phänomen und korreliert normalerweise mit einem invasiven Charakter des Tumors. Interessanterweise entwickelte sich in unserem Modell kein invasiver Phänotyp, was darauf schließen lässt, dass das Verlorengelassen von glatter Muskulatur alleine nicht ausreicht, um zu einem invasiven Tumor zu führen.

Alles in allem bietet diese Studie neue Einblicke in die Zusammenhänge von Entzündung und Prostatakrebsentstehung in einem bereits neoplastisch transformierten Gewebe.

### **3 Introduction**

#### **3.1 Anatomy of the prostate gland**

Generally the prostate consists of several epithelial glands and a fibromuscular stroma. Three different types of cells can be found in this glandular epithelium: basal, luminal secretory and neuroendocrine cells. The role of the basal cells is not fully understood yet but they seem to secrete certain components of the basement membrane and a specific subset of these cells might be epithelial stem cells. The luminal cells are responsible for the production of components of the prostatic fluid, expression of the androgen receptor and secretion of prostate – specific antigen (PSA) in an androgen – dependent manner. (1)

The stroma consists of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating immune cells. Some stromal cells are responsive to androgen and produce growth factors, which in turn interact with the epithelial cells in a paracrine fashion. This stromal – epithelial interaction plays a very important part in the development and maintenance of the prostate. (2)

While the mouse and human prostate are similar in histological organization, there are some significant differences. The murine prostate is assembled by four different lobes: the dorsal, lateral, ventral and anterior lobe. In comparison, the human prostate does not feature four distinct lobes but three different zones: the central, peripheral and transitional zone. (*Figure 1*) Notably, around 70% of human prostate cancers arise in the peripheral zone. (3)

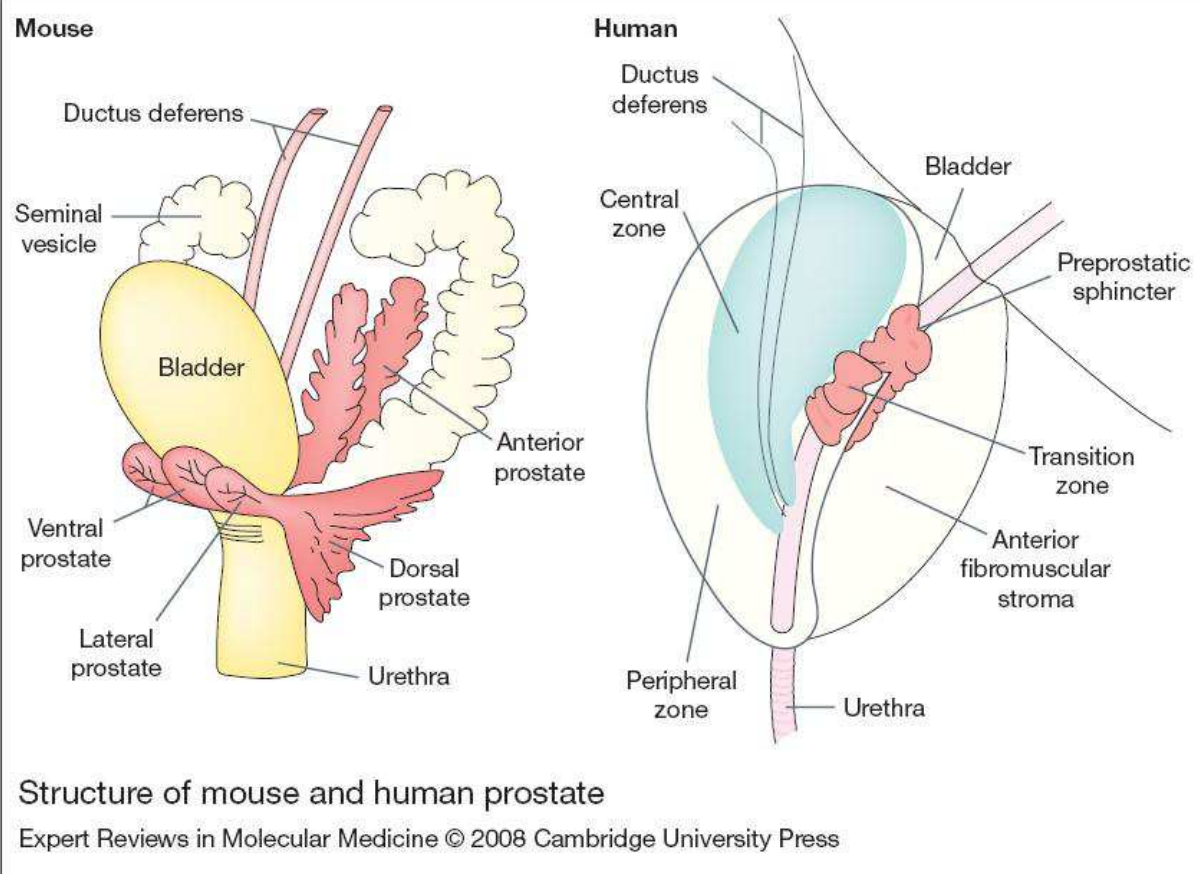


Figure 1: Comparison of a mouse (left) and a human (right) prostate. Source: (3)

## **3.2 Cancer**

Cancer is the leading cause of death in the Western world and the second leading cause of death in developing countries. Estimates for the year of 2008 add up to 12,7 million cancer cases and 7,6 million cancer deaths worldwide. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females, both in developed and developing countries. Prostate cancer is the most prevalently diagnosed cancer among males in developed countries and the third leading cause of cancer related death. In developing countries prostate cancer ranges on fifth place both in incidence and cancer related death. This results in prostate cancer being the second most commonly diagnosed cancer and the sixth leading cause of cancer related death in males worldwide. (4)

In agreement with the above data prostate cancer is the most frequently diagnosed cancer in Austria, accounting for around 24% of diagnosed cancer incidences per year. Since the 1980ies prostate cancer was on a steady rise, which peaked in 2004 with nearly 5700 reported cases. The dramatic increase in the 1990ies can be explained by the massive implementation of PSA – screens, which have been performed on a regular basis since then. The mortality rate of prostate cancer in Austria was decreasing slightly since 2000 after remaining at a steady state for the past decade. (5)

### **3.2.1 *The hallmarks of cancer***

Tumorigenesis is a multistep process evolving over many years. Thousands of different aberrant genes have been documented in different cancer tissues in the past, which demonstrates the complexity of this disease.

So far, it was believed that only six essential alterations in cell physiology were sufficient to collectively lead to malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. (6) However, in the last few years several studies indicate an increasingly important seventh player in tumorigenesis: inflammation. (Figure 2) (6,7)

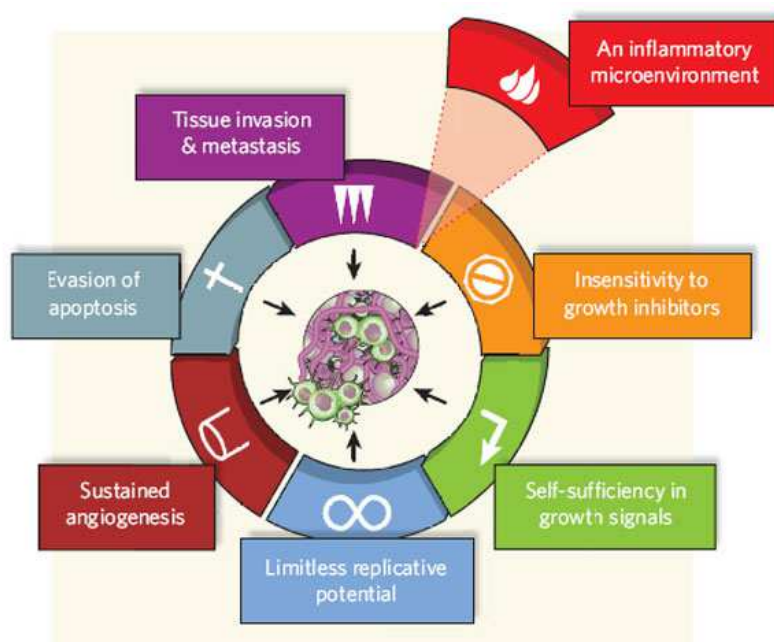


Figure 2: The hallmarks of cancer. Source: (6)

Mitogenic growth factors are a requirement for the proliferative activity of normal cells. Tumor cells on the other hand are far more independent from exogenous growth factors. This leads to the conclusion that tumor cells must be able to produce their own growth factors and thereby reduce the dependency on the cellular microenvironment. This is achieved by oncogenes feigning normal growth signaling in several different ways. (6)

Under normal circumstances antiproliferative signals are responsible for the maintenance of cellular quiescence and tissue homeostasis. If cancer cells are to expand they have to elude these growth inhibitors. Reactivity to antigrowth factors is closely linked to the G1 phase of a cell's growth cycle. The retinoblastoma protein (pRb) pathway plays a major role in the progression from G1 into S phase and thus the disruption of this pathway leads to insensitivity to antigrowth factors. This can be achieved in various ways, for example, by downregulation of the receptors of a signaling molecule or the expression of aberrant receptors. (6,8)

Damaged or otherwise aberrant cells typically undergo programmed cell death in a normal tissue in order to prevent them from causing any damage. In order to grow tumor cells must not only be able to proliferate independently of the cellular environment, they also have to avoid apoptosis. The most commonly found way in tumors to acquire resistance to apoptosis is the mutation of the tumor suppressor p53, which plays an important role in the DNA damage sensor machinery that can induce apoptosis. (6)

Interestingly, self – sufficiency in growth signals, insensitivity to growth inhibitors and evasion of apoptosis is still not enough for a tumor to develop. Most of the mammalian cells exhibit certain mechanisms which prevent cells from dividing endlessly. For example, with each completed replication cycle the telomeric ends of a chromosome shorten which makes it necessary to limit the number of possible cell divisions. If replication continues the ends of the chromosome cannot be protected anymore and the cell dies. To counter this, the most commonly found mechanism in tumors cells is the upregulation of the telomerase enzyme. (6,9)

Furthermore, for any cell to survive it needs to be supplied with oxygen and nutrients. This is the responsibility of the vasculature, whose development is tightly regulated. In order for a tumor to be able to keep growing it has to induce and sustain angiogenesis. To this end tumors activate an angiogenic switch by interfering with the balance of angiogenic inducers and inhibitors. (6)

These five hallmarks of cancer are not only required for a primary tumor to grow and survive but also for successful tumor invasion and metastasis, which constitutes the sixth hallmark of cancer and can be held responsible for around 90% of human cancer deaths. (6,10)

As already mentioned above, nowadays inflammation is considered the seventh hallmark of cancer. Nearly all cancer types can be associated with inflammation in one way or another. However, the exact role inflammation plays in cancer development and progression seems to be very complex and is still subject of extensive study. (7,10)

### 3.3 Prostate Cancer

As previously stated prostate cancer is one of the leading causes of cancer related deaths worldwide. Just like any other type of cancer, prostate cancer is subject of aforementioned conditions.

#### 3.3.1 Genes involved in prostate cancer

Recent studies have identified the most commonly altered genes in prostate cancer. These include, but are not limited to, AR, PTEN, Nkx3.1 and TP53. (Figure 3) (11,12)

	Hypermethylation of GSTP1	Loss of NKX3.1 expression (haploinsufficiency?)	Mutations in PTEN	Mutations in TP53	Mutations in AR	Amplification of AR gene
Normal prostate or BPH	0%	5%				
PIA	5-10%					
PIN	70%	22%				
Localized prostate cancer	90-95%	5-10%	2-15%	5-15%	~0%	0%
Metastatic and/or hormone-refractory prostate cancer		30-80%	10-60%	20-40%	5-25%	30%

Figure 3: The most commonly mutated or epigenetically altered genes found in prostate cancer. Percentages indicate the frequency of appearance in different stages of the disease. Source: (11)



### **3.3.1.1 Androgen Receptor (AR)**

All types of cancer depend on increased cell proliferation and reduced cell death in order to be able to grow. In the prostate androgens are responsible for the regulation of the proportion of cell proliferation and apoptosis by, on the one hand, activating proliferation and on the other hand repressing apoptosis. The main circulating androgen is testosterone, which is converted into the more active dihydrotestosterone (DHT) upon arrival in the prostate. DHT has a fivefold higher affinity to the androgen receptor. Testosterone and DHT bind to the AR in order to induce transcriptional activity. This process is regulated by interaction of AR with coregulators and by phosphorylation of AR or the cofactors in response to various growth factors. (6,2)

A functional AR is required for the initial development of the prostate as AR knockout experiments have shown. After the development of the prostate androgens and AR are important for the maintenance of the secretory epithelia, by balancing cell survival and death. Approximately 80-90% of prostate cancers are dependent on androgen at initial diagnosis. Interestingly, at some point in prostate cancer formation an androgen independency arises. AR expression, however, persists throughout prostate cancer progression. This is an important point as a very commonly used treatment against prostate cancer includes androgen ablation therapy. Studies have shown that AR mutations are rather rare in untreated prostate cancers. Therefore it has been suggested that other signaling pathways are interacting with AR and thus promoting carcinogenesis. Moreover it is hypothesized that an altered gene expression of the various coregulators of AR plays an important role in prostate tumorigenesis. (2)

#### **3.3.1.2 Phosphatase and Tensin Homolog (PTEN)**

The tumor suppressor gene PTEN has been found to be frequently mutated in glioblastoma, breast and prostate cancers. PTEN is a lipid phosphatase that removes the 3 – phosphate from 3 – phosphorylated inositol lipids. These lipids function as second messengers that lead to the activation of the protein kinase AKT or protein kinase B (PKB). AKT is known to phosphorylate and inactivate several proapoptotic genes, which is important for the balance of cell proliferation and death. Thus, by mutating PTEN and therefore activating the AKT pathway cells are able to avoid apoptosis. Moreover, AKT has also been shown to be involved in cell cycle progression. (11)

#### **3.3.1.3 NKX3.1**

The human homeobox gene NKX3.1 is required for normal development of the human prostate, where it is heavily involved in the formation of the prostatic epithelium. Furthermore it is needed for normal prostate function as null mutants display defects in ductal morphogenesis and secretory protein production. NKX3.1 also regulates the proliferation of the prostate epithelium. Loss of NKX3.1 is frequently detected in human prostate cancer and has been associated with hormone – refractory disease and advanced tumor stage. (11,12)

#### **3.3.1.4 TP53**

The tumor suppressor gene TP53 encodes for the tumor suppressor gene p53, which is heavily involved in the regulation of the cell cycle. P53 is the most frequently mutated gene in human cancers. (11)

### **3.4 Inflammation**

Generally, inflammation is an elementary immune response that is important during injuries or infections. Under these circumstances inflammation can secure survival and maintain tissue homeostasis. The downside of inflammation is, however, that it can lead to a transient decline in tissue function, which in turn can contribute to the pathogenesis of various diseases. (13)

Typically, an inflammatory response is made up by inflammatory inducers, sensors, inflammatory mediators and the target tissue. The number of possible players of an inflammatory response is huge and strongly depends on the type of infection or injury. For example, while bacterial infections lead to a macrophage induced production of certain chemokines (CCL2, CXCL8) and cytokines (TNF, IL-1, IL-6) and viral infections lead to the production of type – I interferons (IFN- $\alpha$ , IFN- $\beta$ ) by the infected cells, infections with parasitic worms lead to the production of different cytokines (IL-4, IL-5, IL-13) by mast cells. All these mediators have a different impact on the course of the inflammatory response and on the subsequent activation of associated signaling pathways. (14)

#### **3.4.1 *NF $\kappa$ B***

NF $\kappa$ B plays a central role in basically all types of inflammation. Moreover, NF $\kappa$ B activity can be detected in many different types of cancer. Generally, these transcription factors regulate the balance between cell survival and death and play a role in the regulation of cell proliferation and differentiation.

NF $\kappa$ B belongs to the rapid acting primary transcription factors. It is present in cells in an inactive state at all times and can be activated without the need for protein synthesis. This is achieved by I $\kappa$ B binding to NF $\kappa$ B in the cytoplasm and thus inactivating it. In order to activate NF $\kappa$ B, I $\kappa$ B needs to be degraded. To this end I $\kappa$ B is phosphorylated by either of the two kinases IKK $\alpha$  or IKK $\beta$ . The binding of certain cytokines, such as IL-1 or TNF $\alpha$  leads to the activation of these kinases via certain adapter molecules. Once I $\kappa$ B is degraded, NF $\kappa$ B can move into the nucleus and impact gene expression. (Figure 4) (14,15)

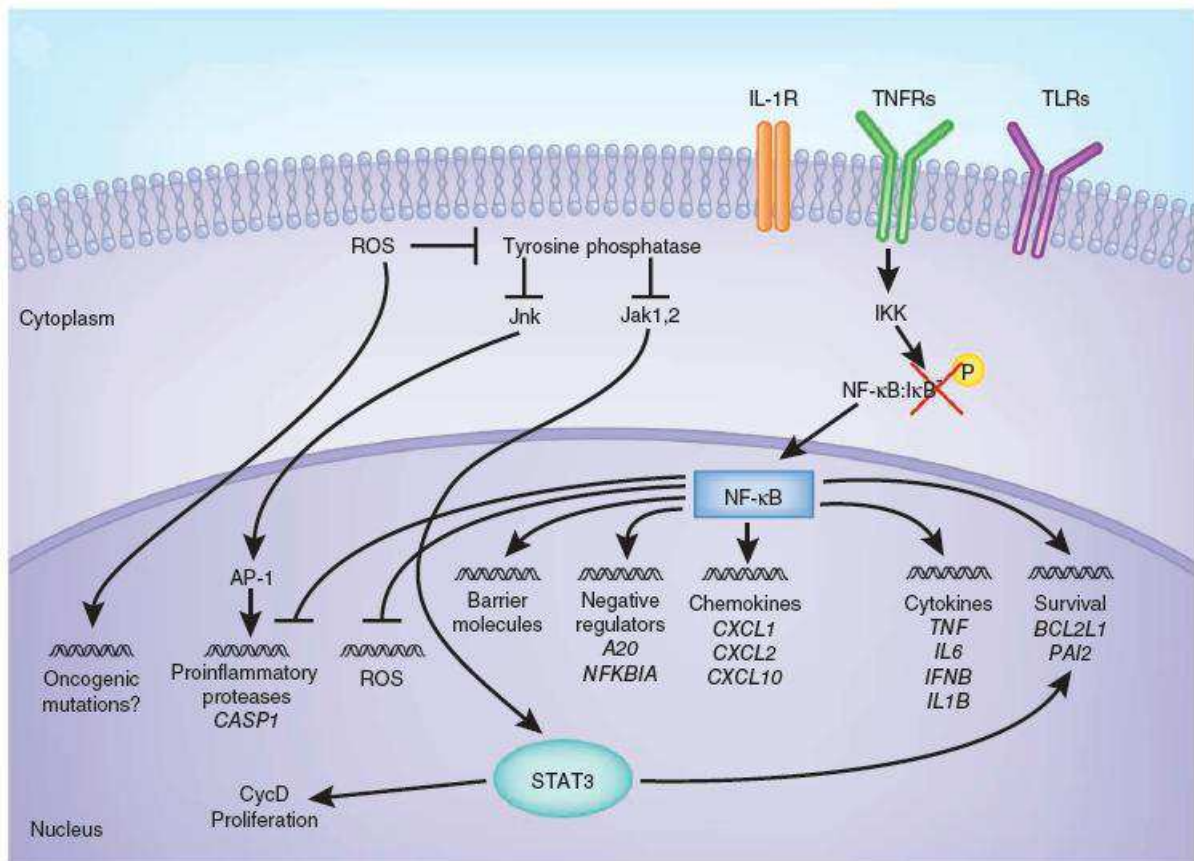


Figure 4: Activation of NF- $\kappa$ B and subsequent induction of several downstream elements playing an important role in tumorigenesis. Source: (14)

### 3.5 Cancer & Inflammation

Inflammation is a very complex disease featuring many different causes, mechanisms, outcomes and intensities. Consequently, different types of inflammation can promote cancer development and progression in different ways.

There are two pathways linking inflammation and cancer: an extrinsic and an intrinsic pathway. The extrinsic pathway is distinguished by inflammatory conditions that increase cancer risk, while the intrinsic pathway is branded by genetic alterations that lead to inflammation and subsequently neoplasia. In the end, both pathways converge and lead to the activation of some consequential transcription factors, mainly NF $\kappa$ B, STAT3 and HIF1 $\alpha$ . These transcription factors are responsible for the expression of inflammatory mediators, which in turn lead to cancer – related inflammation via various mechanisms. (*Figure 5*)

(16)

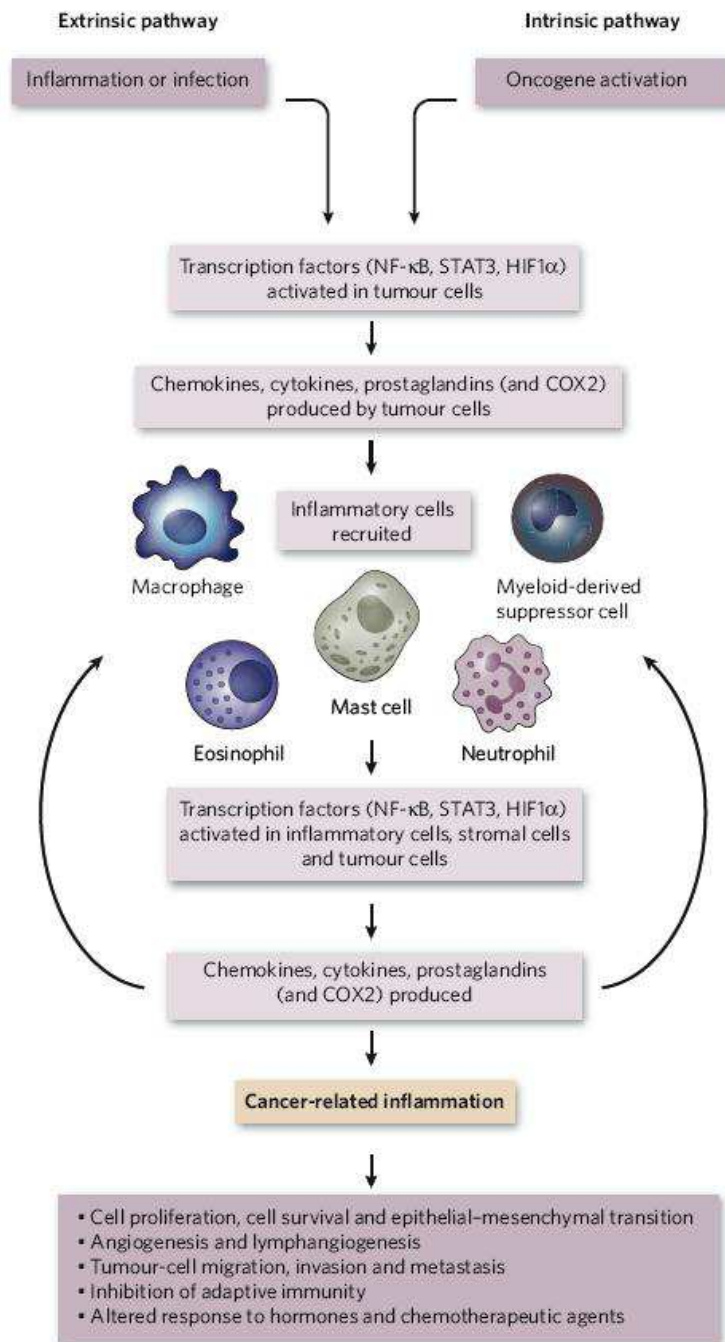


Figure 5: Two pathways link inflammation and cancer: the intrinsic and extrinsic pathway. While they are activated by different causes, they subsequently converge, resulting in the activation of certain transcription factors. This results in the induction of specific cytokines and chemokines and in the end in cancer related inflammation with severe implications on tumor development. Source: (16)

The correlation of various infections and specific types of cancer is well documented. Generally, an infection leads to an immediate inflammatory response as part of the normal defense system of the host. Unfortunately however, tumorigenic pathogens are able to form persistent infections. These infections go along with chronic inflammation which in turn favors tumor development and progression. Another cause of chronic inflammation can be immune deregulation and autoimmunity. (10)

Furthermore, several studies have shown that the exposure to environmental factors such as tobacco smoke, which can be linked to lung cancer, or asbestos or even obesity plays a huge role in tumor formation and progression, as it can also lead to chronic inflammation. (10,17)

All these types of inflammation precede and promote tumor formation in one way or another. In contrast, there is also inflammation that follows tumor development, constituting a protumorigenic microenvironment. This can be induced by certain oncogenes, such as RAS and MYC, whose activation leads to an alteration of the tumor microenvironment through the recruitment of leukocytes and lymphocytes, expression of tumor – promoting cytokines and chemokines and induction of an angiogenic switch. Furthermore, if a tumor is lacking oxygen or nutrients necrotic cell death will occur at its core. This leads to the release of pro – inflammatory mediators, which in turn promotes neo – angiogenesis and facilitates additional growth factors for the tumor cells. (10,16) Moreover, some tumors are able to produce an inflammatory microenvironment by actively secreting certain molecules, such as IL1 $\beta$ .

Interestingly, cancer therapy can be another strong elicitor of tumor associated inflammation. Both radiation and chemotherapy lead to a huge amount of necrotic cell death, which in turn results in an inflammatory response, as already mentioned above. (10)



Due to inflammation the tumor microenvironment consists of both cells of the innate and adaptive immune system, apart from cancer cells and the encased stroma. Distinct communication of these different cell types with each other is essential for the maintenance of tumor growth. They can either interact directly or via the expression of cytokines and chemokines. This composition of immune modulators and the state of activation of different cell types is exactly what makes the distinction between tumor promoting inflammation and anti tumor immunity.

Many cells found in the microenvironment have both tumor promoting and inhibiting properties. The most commonly found cells are tumor associated macrophages (TAMs). TAMs generally promote tumor growth and stimulate T – cells, which can be divided into various subsets with different consequences on said tumor growth. Interestingly, similar T – cell subsets may act pro – tumorigenic in one and anti – tumorigenic in another type of cancer. The exact reasons for this still remain unknown and will need to be elucidated in the future. (10,18)

However, probably more important than the actual composition of immune cells in the tumor microenvironment is the chemokine and cytokine expression profile. Cytokines lead the activation of different downstream regulators, such as NFkB, STAT, AP-1 or SMAD, regardless of their source. Therefore they control the immune and inflammatory milieu to either lead to the formation of a microenvironment favoring anti – tumor immunity or endorse tumor progression. Moreover, certain cytokines can also have direct influence on cell growth and survival. (10)

### **3.5.1 Tumor initiation**

The role of inflammation in tumor initiation is again not clearly elucidated yet. Tumor initiation starts with a single mutation which provides a cell with survival advantages and is typically followed by additional mutations. The cause of this / these mutation(s) might be found in environmental mutagens but there is also the possibility that inflammation is much more critical in this process than previously anticipated. On the one hand activated inflammatory cells constitute a source of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), which can both lead to DNA damage. On the other hand it would also be possible that immune cells utilize specific cytokines to excite an aggregation of ROS. (6,10)

Furthermore, inflammation induced mutagenesis might lead to the inhibition of mismatch repair enzymes and subsequently the inactivation of important tumor suppressors.

The upregulation of the enzyme AID (activation – induced cytidine deaminase), which is known to be up regulated in many cancers, is another possibility to associate inflammation with oncogenic mutations. AID promotes immunoglobulin gene class switching and its production either induced by inflammatory cytokines in an NF $\kappa$ B dependent manner or TGF $\beta$ . AID expression leads to an increased genomic instability and mutation chance. (10,19)

Several alternatives have been proposed in the past years how inflammation could correlate with tumor initiation. There have been hints, for example, that inflammation induced epigenetic mechanisms might play a role in tumor initiation in one way or another. Another proposed mechanism includes copying a stem cell like phenotype on progenitor cells or exciting stem cell expansion by the production of certain growth factors and cytokines. (10)

### **3.5.2 Tumor promotion**

There are many different ways how inflammation can affect tumor promotion. Certain chemokines and cytokines, produced by immune and inflammatory cells play a very important role in tumor promotion. These tumor promoting cytokines lead to the activation of certain transcription factors, such as NFkB, STAT3 and AP-1 which in turn results in the induction of genes for cell proliferation and survival. The target genes of these transcription factors exerting tumor promoting functions are numerous and still subject of study.

Tumor promoting cytokines might also be very important for the promotion of angiogenesis in hypoxic regions. There have been hints that hypoxic stimuli might generate inflammatory signals, which in turn promote angiogenesis. This would be supported by the fact that many pro – angiogenic genes are under direct control of NFkB, STAT3 and AP-1 in TAMs and other cell types. (10)

### **3.5.3 Metastasis**

Over 90% of cancer deaths can be accounted to metastasis. Recent studies have shown that successful metastasis depends on distinct cooperation between cancer and inflammatory cells, as well as stromal elements. (6,10)

Generally, four major steps are required for the process of metastasis. First, cancer cells need to acquire fibroblastoid characteristics, which lead to an increased mobility and the ability to invade epithelial membranes. To this end they need to undergo epithelial – mesenchymal transition (EMT). An important regulator of EMT and metastasis is TGFβ, whose function is quite diverse in a tumor microenvironment and seems to be tightly regulated. Other regulators of EMT are Twist and Kiss, whose transcription is activated by STAT3 and NFkB

signalling. Snail, a repressor of E – Cadherin is stabilized in the presence of TNF $\alpha$  and therefore constitutes another important player in EMT. (10,20)

Secondly, cancer cells intravasate into blood vessels. This step might be driven by inflammation and the production of certain mediators, which increase vascular permeability. For example, IL-1, TNF $\alpha$  and IL-6 seem to promote invasiveness by NF $\kappa$ B and STAT3 mediated expression of certain matrix metalloproteinases (MMP), which are essential for the degradation of the extracellular matrix. In prostate and breast cancers accumulation of activated IKK $\alpha$  is related to reduced expression of maspin, which is a known inhibitor of metastasis. The exact nature of this mechanism, however, is not yet fully understood. (10,21)

Thirdly, the metastasis initiating cells surviving the preceding steps start to travel throughout the circulation. Specific inflammatory mediators, such as TNF $\alpha$  and IL-6, are required for these cells to survive in suspension and resist detachment – induced cell death. On the one hand they lead to NF $\kappa$ B and STAT3 activation, on the other hand some cytokines are also able to physically link cancer cells to TAMs, resulting in them travelling together throughout the circulation. Moreover, this link also provides a certain protection from the immune system.

The final step is represented by single metastatic progenitors interacting with local inflammatory and stromal cells and starting to proliferate. Systemic inflammation alleviates the attachment of circulating cancer cells to certain tissues, as several proinflammatory cytokines lead to an upregulation of the expression of certain adhesion molecules. (10)

### **3.6 Inflammation and prostate cancer**

People suffering from chronic prostatitis are known to have an increased chance of developing prostate cancer at some point. The postulated precursor of prostate cancer is high – grade prostate intraepithelial neoplasia (HGPIN), which in turn some researchers believe to develop from proliferative inflammatory atrophy (PIA). Interestingly, the atrophic epithelial cells in PIA show increased proliferation compared to normal cells. Moreover, PIA, HGPIN and carcinoma feature several similar molecular and genetic changes, such as chromosome 8 abnormalities, p53 mutations, an increased level of Bcl-2 protein and downregulation of PTEN. Furthermore, morphological studies have come to the conclusion that there is a direct transition between PIA, HGPIN and carcinoma. All this leads to the conclusion that the atrophic epithelial cells in PIA might be the target of neoplastic transformation and thus induce carcinoma formation either directly or indirectly via HGPIN development. (22,23)

In most prostate cancer incidents the exact cause of inflammation remains unknown. As previously illustrated, the potential causes range from direct infection and dietary factors to an autoimmune reaction or – specific for the prostate – urine reflux. (10,23)

### **3.7 Aim of the study**

As already mentioned prostate cancer is the most frequently diagnosed cancer in males in the Western world and is also on the rise in developing countries. It is one of the leading causes of cancer related deaths worldwide. Although several theories about the exact mechanisms behind this disease have been proposed so far, there is still a lot of work to do to validate or falsify these hypotheses.

In the last years inflammation has been confirmed to play an important role in various cancer types and is considered a hallmark of cancer by now. There is a growing amount of evidence that inflammation is also needed for the development and progression of prostate cancer. It is not only essential to elucidate the role of inflammation in order to finally understand the underlying causes of development and progression of prostate cancer but certain key players in inflammatory signaling could also constitute promising targets for anti cancer therapies.

In this study we wanted to deepen our understanding of how inflammation can promote prostate tumorigenesis and possibly identify new players involved in the process. To this end we used one transgenic mouse model, which featured a heterozygous knockout of the tumor suppressor PTEN in prostate epithelial cells and another one featuring a constitutively active IKK2. By crossing these two we received a double transgenic mouse model featuring PIN formation and thus the initial step of prostate tumor formation combined with an inflammatory microenvironment. Therefore, we were able to compare morphology and gene expression of certain genes of the double transgenic mice with mice carrying only the PTEN knockout and wildtype mice. This enabled us to investigate the impact of a chronic inflammation on tumor development in a prostate with neoplastic transformation.

Starting with simple HE stainings to illustrate the morphological changes in the genetically different prostates, we moved on to staining the tissues with specific immune and proliferation markers and studied the gene expression of selected genes via RT – PCR.

## 4 Results

### 4.1 Strong stromal response in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates

The monoallelic deletion of PTEN leads to the development of prostatic intraepithelial neoplasias (PIN) late in mouse life. (24) We wanted to know if a constitutively active IKK2 signaling would influence this tumor formation and whether it would promote invasiveness. To this end we performed histological stainings using Hemalaun and Eosin. Hemalaun stains the nuclei and the ER blue while Eosin stains the cytoplasm and collagen fibers pink. These stainings revealed that wildtype tissue showed a normal epithelium while the PTEN<sup>+/-</sup> prostate had some hyperplasia and PINs. The epithelium of PTEN <sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates emerged as much more hyperplastic but with the same amount of nuclear atypia as PTEN<sup>+/-</sup> mice. Interestingly the stroma of the double transgenic mice appeared to be highly altered. It clearly contained several collagen fibers compared to both wildtype and PTEN<sup>+/-</sup> mice. (*Figure 1*)



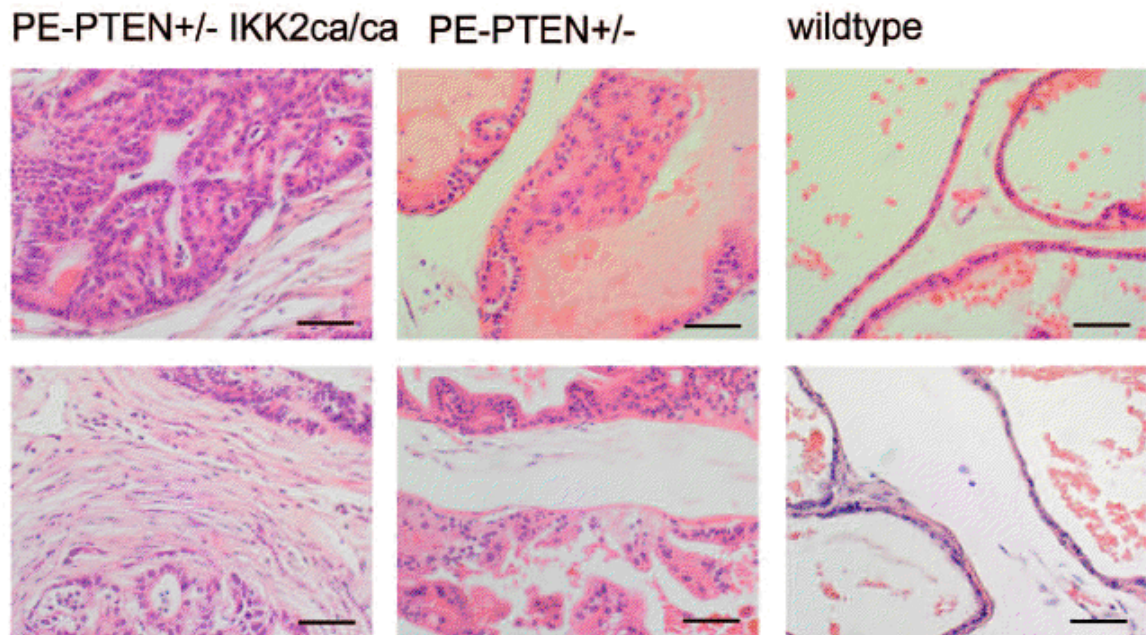


Figure 3: Histological staining of PTEN<sup>+/-</sup> IKK2ca/ca, PTEN<sup>+/-</sup> and wildtype prostates; upper panel: the epithelium of a PTEN<sup>+/-</sup> IKK2ca/ca prostate appears as severely hyperplastic compared to the PTEN<sup>+/-</sup> prostate, which is less hyperplastic, and the wildtype prostate. Lower panel: the stroma of the PTEN<sup>+/-</sup> IKK2ca/ca prostate appears highly altered containing numerous collagen fibers in comparison to the wildtype and PTEN<sup>+/-</sup> prostate.

These data show that, apart from the appearance of hyperplasia and the formation of PINs, there is a strong stromal response in PTEN<sup>+/-</sup> IKK2ca/ca prostates compared to both PTEN<sup>+/-</sup> and wildtype prostates. This is an interesting finding as interactions between the epithelium and the stroma play an important role in prostate development and disease.

#### **4.2 Increased proliferation in PTEN<sup>+/-</sup> IKK2ca/ca mice compared to PTEN<sup>+/-</sup> and wildtype mice**

Aside from the strong stromal response and the occurrence of PINs we also found an increase in proliferation of both epithelial and stromal cells in PTEN<sup>+/-</sup> IKK2ca/ca mice compared to PTEN<sup>+/-</sup> and wildtype mice.

Nuclear Ki67 is known to be solely expressed in proliferating cells and is therefore commonly used as a target for antibodies in cancer diagnostics to check for an increase in proliferation in different tissues. (25) Thus we stained different tissues with Ki67 antibody and counterstained with Hoechst. Hoechst binds to the minor groove of the DNA and therefore stains all cells. This is important in order to be able to eliminate false positive and background signals. While there is little to no positive Ki67 signal to be found in the PTEN<sup>+/-</sup> and wildtype prostates several positive signals are visible in the PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates indicating increased proliferation in this tissue. (Figure 2)

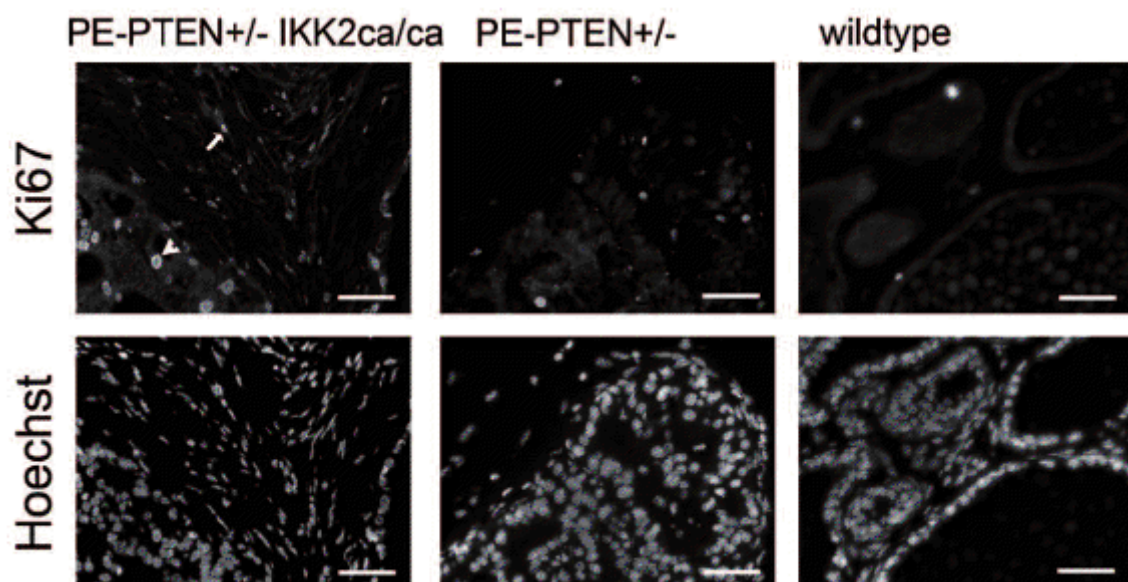


Figure 2: Immunofluorescence staining of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup>, PTEN<sup>+/-</sup> and wildtype mouse prostate tissues with the proliferation marker Ki67; arrows indicate positive signals; counter stained with Hoechst

One basic requirement for a tumor to be able to grow and prosper is the deregulation of cell proliferation. (26) Furthermore, being independent of growth signals and immune to anti – proliferative signals are two hallmarks of cancer. (6) To this end the increased proliferation in our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> mice is a very interesting thing to note.

### **4.3 Persistent Inflammation in PTEN+/- IKK2ca/ca prostates**

In addition to the increased proliferation in the epithelium and stroma of PTEN+/- IKK2ca/ca prostates and the general stromal response there were also clear signs of a severe prostatitis. Prostatitis is the histological inflammation of the prostate, which is indicated by the infiltration of the tissue by immune cells. One of the first cells to arrive at the target site are neutrophils. Their function spans from the recruitment of other immune cells to the isolation and elimination of pathogens. (27) The other quickly arriving type of immune cells are monocytes. On the one hand monocytes can actively purge infected cells or pathogens and on the other hand they can differentiate into either dendritic cells or macrophages which in turn hold several different functions including phagocytosis. (28)

To check for the presence of these cells we performed immunofluorescence stainings with CD11b, F4/80 and GR-1 antibodies. CD11b is a macrophage and monocyte specific antibody while F4/80 stains macrophages only and GR-1 specifically binds to neutrophils. Again, we counterstained with Hoechst in order to be able to filter out any false positive signals. We were able to prove the presence of all of these three markers in our PTEN+/- IKK2ca/ca prostates. (*Figure 3*)

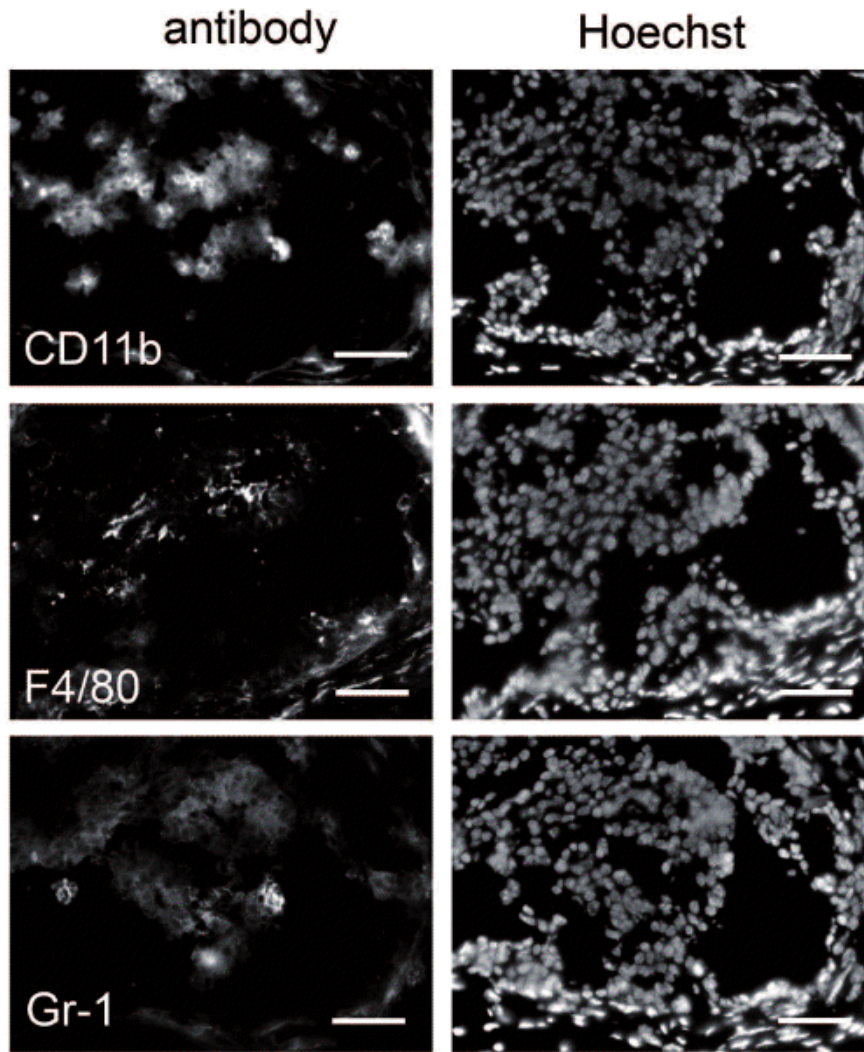


Figure 3: Immunofluorescence staining of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostate tissues with the immune cell markers CD11b (monocytes & macrophages), F4/80 (macrophages) and GR-1 (neutrophils); counter stained with Hoechst

These data clearly show that there is a persistent inflammation going on in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates. The constitutive activation of IKK2 in PTEN<sup>+/-</sup> prostates is enough to lead to severe inflammation. As inflammation has emerged as a very important player in cancer development and progression in the last years this mouse model appears to be perfect to study the connection between inflammation and prostate cancer. (7)

#### 4.4 Loss of smooth muscle in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates

To further investigate the reasons behind the changes in the stroma of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates revealed by our histological stainings we next checked the expression of several stromal genes. Interestingly we found that the two smooth muscle cell markers smooth muscle myosin heavy chain (MYH11) and smooth muscle actin (SMA) were drastically downregulated in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates compared to PTEN<sup>+/-</sup> and wildtype prostates. (Figure 4)

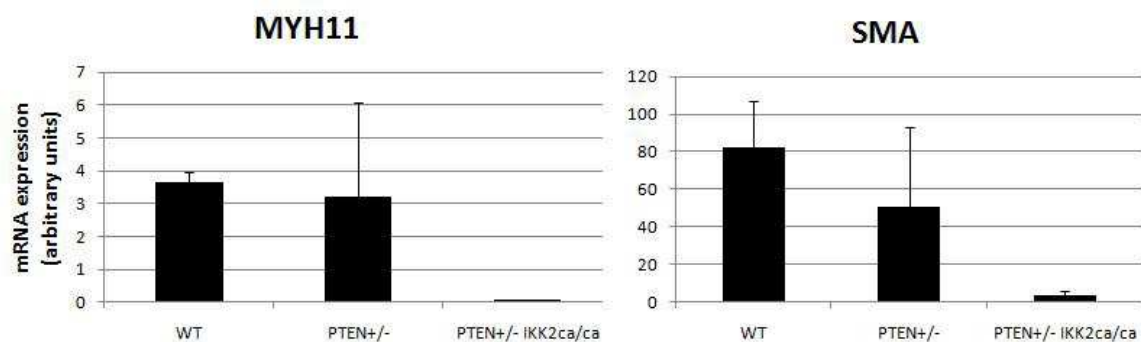


Figure 4: quantitative RT – PCR comparing the expression level of MYH11 and SMA in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup>, PTEN<sup>+/-</sup> and wildtype mouse prostate tissues.

Expression levels of MYH11 were much lower in our double transgenic mice compared to the wildtype and PTEN<sup>+/-</sup> mice. The difference in the expression level of SMA was even more apparent when comparing these three genotypes, accentuated by a 40 to 80 fold downregulation.

To further emphasize these results we also stained prostate tissues with the respective genotypes with SMA antibody. Smooth muscle Actin (red staining) continuously surrounds the prostatic ducts in the PTEN<sup>+/-</sup> and wildtype prostate tissues while there nearly is a complete loss thereof in the PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> tissue. (Figure 5)



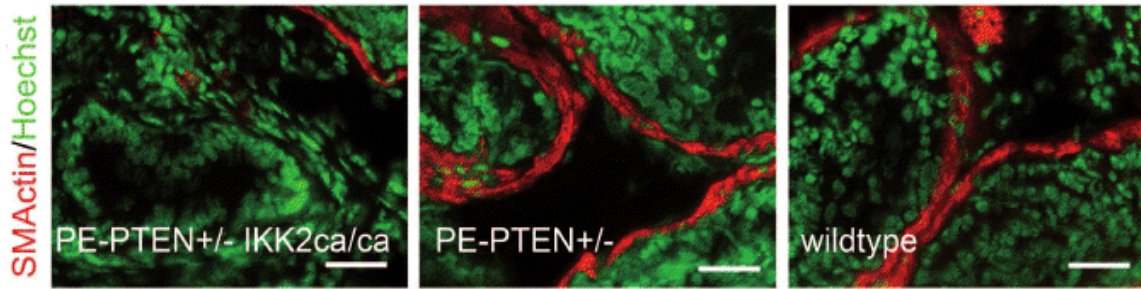


Figure 5: Immunofluorescence staining for SMA (red) on PTEN+/- IKK2ca/ca, PTEN+/- and wildtype prostate tissues; counterstained with Hoechst (green)

Myh11 mutations have been identified in human colorectal cancer and acute myeloid leukemia in the past. Generally Myh11 has been hypothesized to also play an important role in various other types of cancer which perfectly goes along with the data we received in our model. (29)

Taken together these data confirm a drastic change in the stroma of PTEN+/- IKK2ca/ca prostates. These changes go along with a noticeable loss of smooth muscle. However, although the loss of smooth muscle is associated with an invasive phenotype in many tumor models, we did not find any indicator of an increased invasiveness in our model.

#### **4.5 PPM1A is down – regulated in PTEN+/- IKK2ca/ca mice compared to PTEN+/- mice.**

Another interesting gene we found, whose expression appeared to be altered was PPM1A. Nuclear PTEN is known to stabilize the Smad2/3 phosphatase PPM1A by establishing a complex with it. (30)

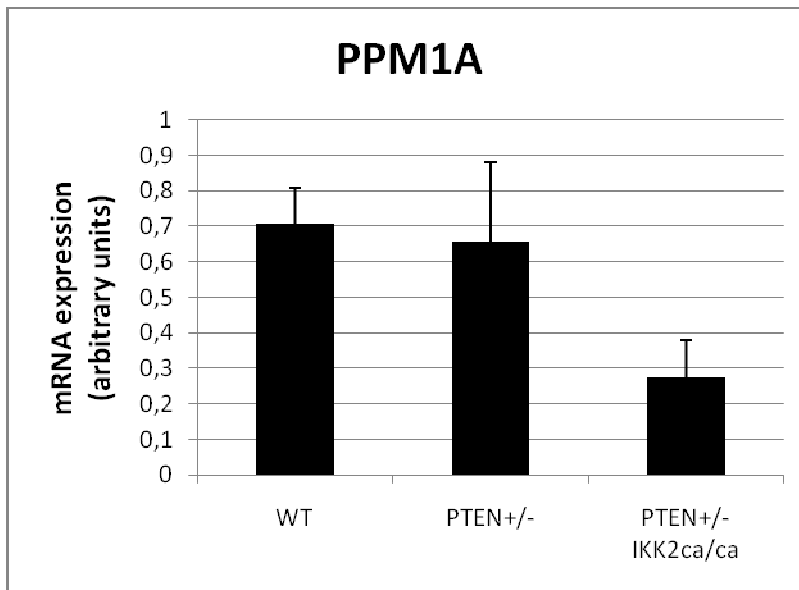


Figure 6: quantitative RT – PCR comparing the expression level of PPM1A in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup>, PTEN<sup>+/-</sup> and wild type mouse prostate tissues

PPM1A expression is slightly down – regulated in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> tissues compared to PTEN<sup>+/-</sup> and wild type tissues. (*Figure 6*)

Since PPM1A is known to interact with PTEN this downregulation appears to be an interesting finding in our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> mouse. However, the exact role of this player and its possible impact on cancer formation and its association with inflammation in our mouse model still remains somewhat unclear and will need to be elucidated in the future.

#### **4.6 Ck18 expression levels are higher in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial cells compared to PE-PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells**

Epithelial – stromal interactions decisively influence the development and functionality of the prostate. Moreover they play a very important role in various derangements of the prostate glands, such as BPH and prostate carcinoma.

(31) In our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates the genetic changes in the epithelium give rise to the inflammation of the whole prostate. For this reason we next wanted to check the gene expression of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial cells against PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal and wildtype cells. Therefore we produced both epithelial and stromal cell lines from a PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> mouse prostate by explant cultures.

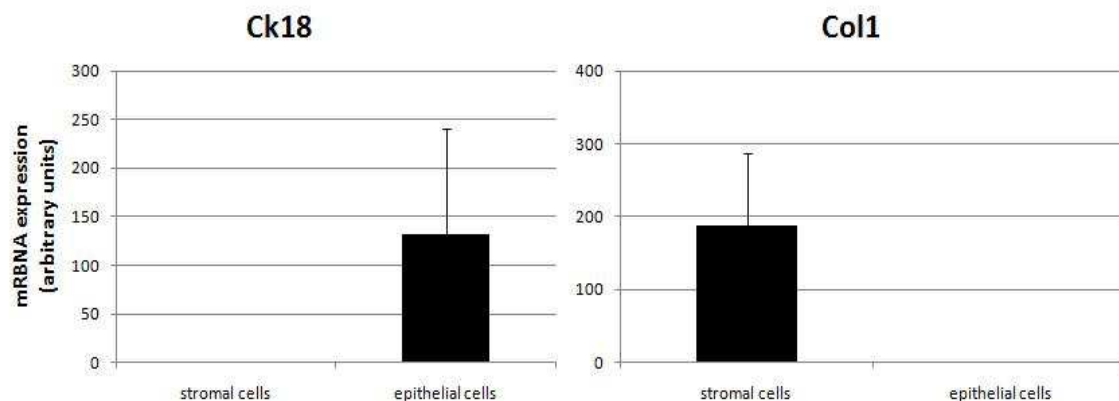


Figure 7: quantitative RT – PCR comparing the expression level of the epithelial marker CK18 and the stromal marker Collagen 1 in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal and PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial cells

Cytokeratin 18 is found in many epithelial tissues. It is hypothesized to have various functions, one being helping to maintain epithelial cell integrity. (32) Therefore CK18 antibodies are frequently used as markers for epithelial tissues in several organs. The CK18 expression in our epithelial cells is hundred fold increased compared to the stromal cells while Collagen I expression, which is a common marker for stromal tissues, is hundred fold increased in the stromal cells compared to the epithelial cells. (*Figure 7*)

These data confirm that the cell lines we established were indeed a stromal and an epithelial cell line, respectively and therefore were perfectly suited to study the different gene expression and the implication diverse chemokines would have on these cell types.



#### 4.7 TNF $\alpha$ expression level is higher in PTEN $^{+/-}$ IKK2 $^{ca/ca}$ epithelial cells compared to wildtype and PE-PTEN $^{+/-}$ IKK2 $^{ca/ca}$ stromal cells

TNF $\alpha$ , a cytokine heavily involved in inflammatory signaling, primary regulator of immune cells, known to play a role in, amongst other diseases, cancer and also known to be a target of NF $\kappa$ B, constituted an interesting target for us to check for an altered gene expression. (33)

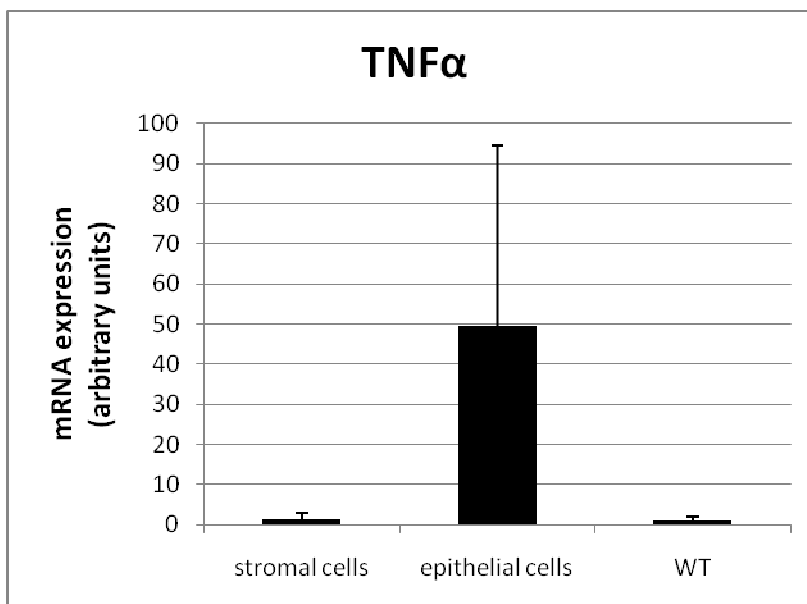


Figure 8: quantitative RT – PCR comparing the expression level of TNF $\alpha$  in PTEN $^{+/-}$  IKK2 $^{ca/ca}$  epithelial and PTEN $^{+/-}$  IKK2 $^{ca/ca}$  stromal and wildtype cells

TNF $\alpha$  expression in both wildtype and PTEN $^{+/-}$  IKK2 $^{ca/ca}$  stromal cells is about 40 fold decreased compared to PTEN $^{+/-}$  IKK2 $^{ca/ca}$  epithelial cells. (Figure 8)

These data show that the constitutive activation of IKK2 in PTEN $^{+/-}$  prostates leads to an increased production of TNF $\alpha$  in the epithelium. This correlation between the altered gene expression in PTEN $^{+/-}$ -IKK2 $^{ca/ca}$  epithelial cells and the subsequently higher expression of TNF $\alpha$  plays an important role in the

recruitment of inflammatory cells and might explain the increased number of immune cells we have proven to infiltrate our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> prostates.

#### 4.8 CXCL15 and CXCL5 expression level is higher in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial cells compared to wildtype and PE-PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells

Generally, CXC chemokines are known to recruit all kinds of immune cells to an inflamed tissue and to be involved in the tumorigenicity of prostate cancer cells. (34) The production of some CXC chemokines can be induced by the stimulation of cells with inflammatory cytokines such as TNF $\alpha$ . (35) This is a very interesting point as we were able to prove that TNF $\alpha$  expression is strongly increased in the epithelium of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> cells.

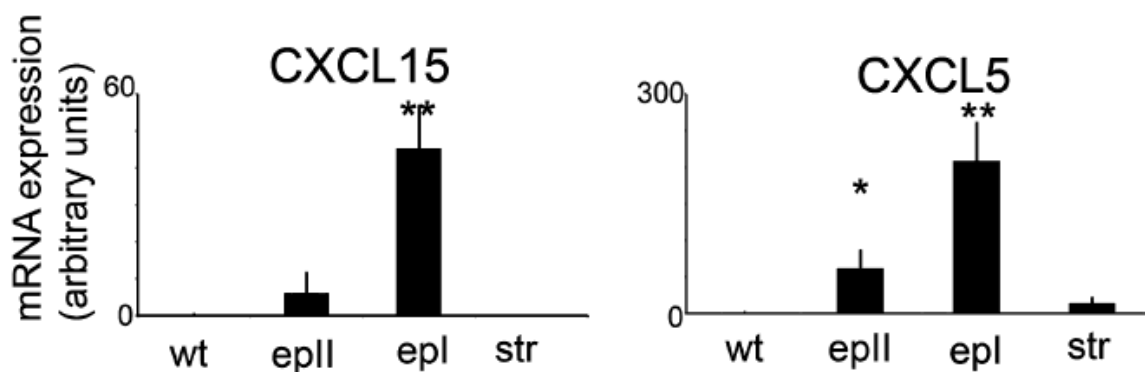


Figure 9: quantitative RT – PCR comparing the expression level of CXCL15 and CXCL5 in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial, PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal and wildtype cells

CXCL15 expression is many times higher in PTEN+/- IKK2ca/ca epithelial cells compared to both PTEN+/- IKK2ca/ca stromal and wildtype cells. CXCL5 is upregulated even more in the PTEN+/- IKK2ca/ca epithelial cells compared to both wildtype and PTEN+/- IKK2ca/ca stromal cells. (*Figure 9*)

CXCL15 was first described in lung epithelial cells as a member of the CXC chemokine family with some peculiar properties. Its primary function is to recruit neutrophils during inflammation. (36)

Similarly to CXCL15, CXCL5 is known to be an activator of the chemotaxis of neutrophils. (37)

These data go in line with the increase of inflammatory cells found in the PTEN+/- IKK2ca/ca tissue. The increased production of both CXCL15 and CXCL5 might explain the increase of immune cells, especially neutrophils, in our PTEN+/- IKK2ca/ca prostate tissue.

#### **4.9 Changes in mRNA expression level of CXCL2 and CXCL10 in PTEN+/- IKK2ca/ca stromal cells after treatment with TNF $\alpha$**

Above, we were able to prove that TNF $\alpha$  expression and the expression of certain CXC chemokines in PTEN+/- IKK2ca/ca epithelial cells was a multiple of the expression in wildtype cells and stromal cells of the same genotype. Moreover, we could show that there is a strong increase of immune cells in PTEN+/- IKK2ca/ca mouse prostate tissues. As already mentioned, epithelial – stromal interactions play an important role in the prostate and its diseases.

Considering all this, we wanted to prove the hypothesis that the stroma, activated by chemokines originating from the epithelium, was also able to recruit immune cells by the production of various chemokines. Therefore we next treated PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells with TNF $\alpha$  in order to see whether this treatment would lead to an altered gene expression in these cells, giving a further explanation for the increased number of immune cells in the stroma.

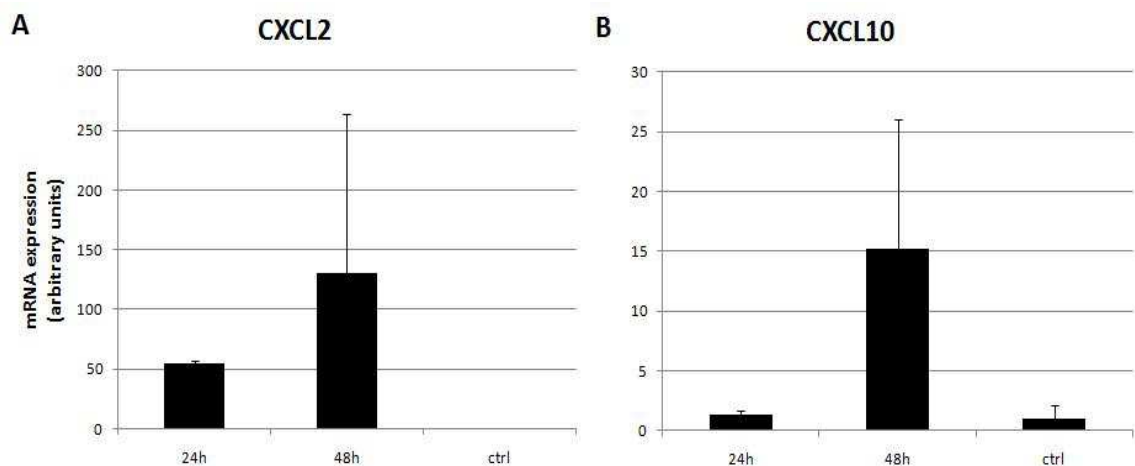


Figure 10: quantitative RT – PCR of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells after treatment with TNF $\alpha$  for 24h and 48h, respectively, comparing the gene expression of CXCL2 (A) and CXCL10 (B) with an untreated control

Already after 24h the expression of CXCL2 in TNF $\alpha$  treated PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells is a multitude of the expression in the untreated control. After incubating the cells for another 24h, the expression levels approximately doubled. The same holds true for the expression of CXCL10. While there is no significant difference in its expression between the untreated cells and the cells incubated with TNF $\alpha$  for 24h, there is a boost of CXCL10 expression after 48h incubation. (*Figure 10*)

CXCL2 is a small chemokine that is secreted by monocytes and macrophages. Its primary function is to recruit immune cells, more precisely granulocytes, to the target site. (38)

The CXC family member CXCL10 has been shown to have several functions such as inhibiting bone marrow colony formation and promoting T – cell adhesion to endothelial cells. But most importantly to us, it acts as a chemoattractant for monocytes. (39)

Taken together these data clearly show that the stimulation of stromal PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> cells with TNF $\alpha$  is enough to induce an increased production of both CXCL2 and CXCL10 in these cells. This, in turn, proves that the increased TNF $\alpha$  expression we detected in our epithelial cells has a huge impact on the gene expression of the two chemokines in the stromal cells. All this supports the hypothesis that the stroma, after being activated by cytokines from the epithelium, can express chemokines which in turn recruit immune cells. This clearly shows that the transgenic PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelium is able to recruit inflammatory cells by either direct or indirect production of chemokines.

#### **4.10 Changes in mRNA expression level of CXCL15 in PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells after treatment with TNF $\alpha$**

As shown above CXCL15 was another member of the CXCL family we found to be upregulated in our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> epithelial cells compared to the PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal and wildtype cells. Therefore we wanted to see if incubation with TNF $\alpha$  would lead to an increased production of CXCL15 in the PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells, just as it turned out to be the case with CXCL2 and CXCL10 as shown before.

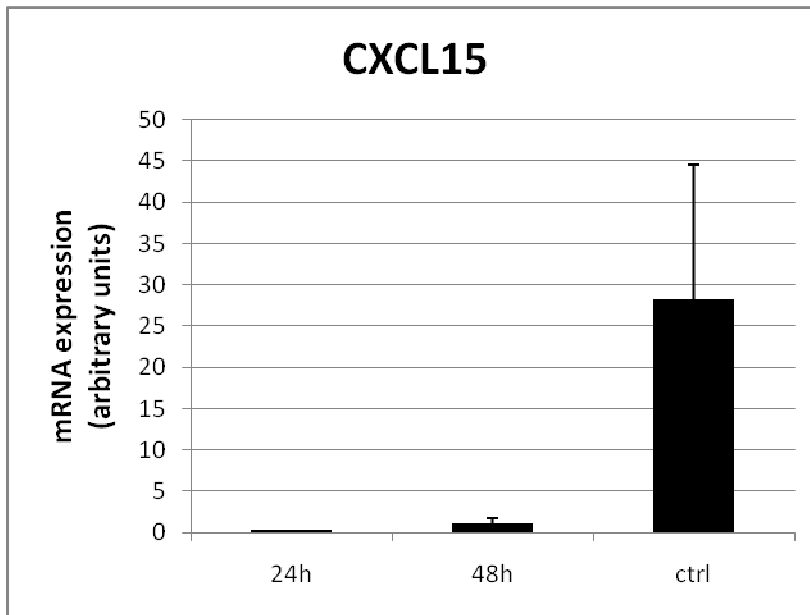


Figure 11: quantitative RT – PCR of PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells after treatment with TNF $\alpha$  for 24h and 48h, respectively, comparing the gene expression of CXCL15 with an untreated control

Interestingly, after treatment with TNF $\alpha$  the stromal cells did not show increased expression of CXCL15 but a strong decrease compared to the untreated control, totally in contrast to CXCL2 and CXCL10. (*Figure 11*)

This is a somewhat interesting finding and will need to be addressed in the future. It could go in line with the fact that CXCL15 has some peculiar properties, as already mentioned above. Another possibility would be that TNF $\alpha$  alone is simply not sufficient to induce CXCL15 expression in our PTEN<sup>+/-</sup> IKK2<sup>ca/ca</sup> stromal cells but needs another co – factor.

## **5 Materials & Methods**

### **5.1 Mice**

All mouse breeding, sacrificing and harvesting of organs was performed in cooperation with Dr. Andreas Birbach in accordance to institutional guidelines.

#### **5.1.1 *Probasin-Cre mice***

Probasin-Cre mice were obtained from the National Cancer Institute – Frederick Mouse Respiratory. Since the Cre gene is under the control of the promotor ARR2PB, which is a derivative of the prostate – specific probasin (PB) promotor, this mouse line features a prostate – specific expression of the Cre recombinase. Originally Cre stems from the bacteriophage P1 and Cre – mediated excision of loxP elements is an essential tool for conditional gene activation or inactivation in molecular biology. (40,41)

#### **5.1.2 *Mice with a floxed PTEN allele***

Mice with a floxed PTEN allele (PTEN<sup>flox/-</sup>) were a gift from Prof. Tak Mak (Ontario Cancer Institute, Toronto, Canada). Crossing of these mice with Probasin-Cre mice leads to the excision of the floxed PTEN allele and therefore results in decreased levels of PTEN. (42)

### 5.1.3 R26StopFIIKK2ca mice

R26StopFIIKK2ca mice were generated by Dr. Marc Schmidt-Supprian (Max Planck Institute for Biochemistry, Martinsried, Germany). After Cre-mediated deletion of the STOP cassette IKK2ca is expressed under control of the endogenous ROSA26 promotor in this strain. (Figure 1) (43)



Figure 1: Scheme of the R26StopFIIKK2ca transgene.

Source: (43)

### 5.1.4 Mouse breedings and genotypes used for this project

The monoallelic deletion of PTEN alone is not enough to transform the prostate but leads to the development of PINs late in mouse life. (24) In order to be able to check whether an additional chronic inflammation of the prostate would lead to the successful transformation of the tissue we established three different mouse lines to compare gene expression and morphology. (Tab. 1)

genotype 1	genotype 2	expected genotype in prostate epithelial cells
Probasin-Cre	R26StopFIIKK2ca	<i>IKK2ca/ca</i>
Probasin-Cre	floxed PTEN allele	<i>PTEN+/-</i>
<i>IKK2ca/ca</i>	floxed PTEN allele	<i>PTEN+/- IKK2ca/ca</i>

Table 1: Genetic background of the mice used in this study. (*IKK2ca/ca*; *PTEN+/-*; *Pten+/- IKK2ca/ca*)



Subsequent analysis of the transgenic mice revealed that the expression level of the flagIKK2 transgene on a PTEN<sup>+/-</sup> background was highest in the lateral prostate. Therefore we used the lateral prostate lobe for all of the experiments in this study.

## **5.2 gDNA**

The respective genotype of each mouse used for this project was confirmed prior to commencing with the experiments. Therefore tail biopsies were taken immediately after sacrificing each mouse. The samples were stored in 500 µl lysis buffer with 3,0 µl Proteinase K (20 mg/ml) added and incubated at 58°C over night.

### **5.2.1 *gDNA isolation***

500 µl 5M NaCl was added to each of the previously incubated samples. After mixing they were centrifuged at 16000g at room temperature for 5 minutes. The supernatant was transferred to a new flask and carefully mixed with an equal volume of Isopropanol. Subsequently the samples were centrifuged at 16000g at room temperature for 30 minutes. After discarding the supernatant and adding 1 ml ice cold 70% Ethanol to each flask, the samples were centrifuged at 16000g at room temperature for 5 minutes. Again the supernatant was discarded and the pellet was air dried for 10 minutes. Finally, the pellet was resuspended in 100 µl 10mM Tris pH8.

### **5.2.2 *Genotyping***

In order to verify the genotype of each sacrificed mouse we performed a Polymerase Chain Reaction (PCR) with the gDNA samples using four different primer pairs. (*Tab. 2*)

Gene / amplicon	Primer sequence (sense / antisense)
CRE	5'-CGGTCGATGCAACGAGTGATGAGG-3' 5'-CCAGAGACGGAAATCCATCGCTCG-3'
PTEN	5'-CTCCTCTACTCCATTCTTCCC-3' 5'-ACTCCCACCAATGAACAAAC-3'
rNeo/IKK2	5'-GAGCTGCAGTGGAGTAGGCG-3' 5'-GCCTTCTTGACGAGTTCTTC-3'
Rosa26	5'-CCAGATGACTACCTATCCTC-3' 5'-GAGCTGCAGTGGAGTAGGCG-3'

Table 2: List of primer sequences used for the PCR

For the PCR 2,5 µl 10 x Buffer (Peq Lab, Erlangen, Germany), 0,5µl of each forward and reverse primer (10 µM), 0,5 µl dNTPs (10 mM), 0,1 µl Taq Polymerase (5 units/µl; Peq Lab) and 20 µl ddH<sub>2</sub>O were added to 1,0 µl of template DNA. The PCR was performed on an eppendorf thermocycler. (eppendorf, Hamburg, Germany) (*Tab.3*)

Step	Temperature	Time	Number of cycles
Initiation	95°C	10 min	1
Denaturation	95°C	30 sec	35
Annealing	55°C	30 sec	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1
Storage	4°C	∞	

Table 3: PCR setup for genotyping

The PCR products were loaded onto an agarose gel to check for the succesful or unsuccessful amplification of the respective genes.

## **5.3 Cell Culture**

Most of the cell culture work was performed in collaboration with Dr. Andreas Birbach.

### **5.3.1 *Explant Culture***

Immediately after harvesting the prostate tissues were cut into small pieces of 0,2 – 1,0 mm and put on collagenated tissue culture plates. A small amount of epithelial growth medium containing 1% serum was added and incubated at 37°C for 24 hours. Once the tissues were attached to the plate more medium was added and then put back into the incubator for another 24 hours. Cells started to grow out from the prostate tissue after another 24 to 48 hours of incubation. In order to obtain either clean stromal or epithelial cell cultures the obverse cell type was removed with a cell scraper or pipette tip. (44)

### **5.3.2 *Cell Passaging***

The media was removed, cells were washed with 1xPBS and an appropriate amount of Trypsin EDTA or Accutase (PAA, Pasching, Austria) was added. Cells started to detach after a short incubation period (5 – 15 min). Subsequently the cells were transferred into a flask, centrifuged, resuspended and put back into the incubator.

### **5.3.3 *TNF treatment of cells***

An appropriate amount of TNF (20 ng/ml; Immunotools, Friesoythe, Germany) was added and cells were incubated for 24 to 48 hours, respectively.

## **5.4 RNA isolation**

Prior to any RNA related work the work bench and all used pipettes were cleaned to be made RNase free.

### ***5.4.1 RNA isolation from prostate tissues***

Harvested prostate tissue was stored in RNAlater (Applied Biosystems, Carlsbad, USA). Tissues were homogenized using a Polytron Homogenizer. The RNA was purified on RNA binding columns using either RNeasy Mini Kit (Qiagen, Hilden, Germany) or peqGOLD Total RNA kit (Peqlab, Erlangen, Germany). The separate steps were performed according to the manufacturer's instructions.

### ***5.4.2 RNA isolation from cells***

The medium was removed, cells were washed with 1 x PBS and 1 ml TRIZOL (Carl Roth GmbH, Karlsruhe, Germany) per 0,1 – 1,0 g of cells was added. The samples were transferred to flasks and incubated at room temperature for 5 – 10 minutes. For complete disruption of the cells the samples were homogenized using a Polytron Homogenizer. 0,2 ml of chloroform for each 1 ml of TRIZOL used in the initial step was added. The samples were vortexed for 15 seconds and incubated at room temperature for 2 – 3 minutes. Then the samples were centrifuged at 12000g at 4°C for 15 minutes. The organic phase and the middle layer were discarded. The aquatic phase, which included the RNA, was transferred into a new flask and 0,5 ml Isopropyl alcohol was added for each 1 ml of TRIZOL used in the initial step. After mixing, the samples were incubated at room temperature for 2 – 3 minutes. This was followed by another centrifugation step at 12000g at 4°C for 10 minutes. The supernatants were

removed and the pellets washed with 1 ml ice cold 75% Ethanol. The samples were then centrifuged at 7500g at 4°C for 5 minutes and the supernatants were discarded. The pellets were air dried for 5 – 10 minutes, resuspended in 10 µl RNase free ddH<sub>2</sub>O and stored at -40°C.

#### **5.4.3 *Measurement of RNA concentration***

RNA concentration was measured using UV spectrometry. Therefore 1 µl of RNA was diluted in 100 µl of RNase free ddH<sub>2</sub>O and 100 µl of RNase free ddH<sub>2</sub>O alone served as blank. The absorbance was measured at 230 nm for contaminations, 260 nm for nucleic acid, 280 nm for protein and 320 nm for the background.

## **5.5 cDNA**

Original RNA samples were kept on ice at all times and after taking an aliquot thawed at -80°C again immediately.

### **5.5.1 DNA digestion**

RNA was diluted in 8 µl RNase free ddH<sub>2</sub>O to obtain a concentration of 0,1 µg/µl. Then 1,0 µl of 10 x DNaseI buffer and 1 µl of DNaseI (Fermentas, St. Leon-Rot, Germany) were added and the samples incubated at 37°C for 30 minutes. The DNase digestion was stopped by incubating the samples at 75°C for 5 minutes.

### **5.5.2 cDNA synthesis**

cDNA was synthesized using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas). The separate steps were performed according to the manufacturer's instructions. Random hexamer primers were used. The produced cDNA was diluted in 40 µl of ddH<sub>2</sub>O and stored at -20°C.

### **5.5.3 Quantitative Real time RT – PCR**

For the quantitative real time RT – PCR 2,5 µl 10 x buffer (Peq Lab), 2,375 µl DMSO (1:100 dilution), 0,125 µl SYBR Green (1:1000 dilution), 0,5 µl of each forward and reverse primer (10 µM) (*Tab.4*), 0,5 µl dNTPs (10 mM), 0,1 µl Taq

Polymerase (5 units/μl; Peq Lab) and 17,5 μl ddH<sub>2</sub>O were added to 1,0 μl of template cDNA.

Gene / Amplicon	Primer Sequence ( sense / antisense)
Ck18	5'-CAGCCAGCGTCTATGCAGG-3' 5'-CTTTCTCGGTCTGGATTCCAC-3'
Col1	5'-CACCCCTCAAGAGCCTGAGTC-3' 5'-GTTCTGGGCTGATGTACCAGT-3'
CXCL2	5'-GCGCCCAGACAGAAGTCATAG-3' 5'-AGCCTTGCCCTTTGTTTCAGTATC-3'
CXCL5	5'-GAAAGCTAAGCGGAATGCAC-3' 5'-GGGACAATGGTTTCCCTTTT-3'
CXCL10	5'-GCTCCTGCATCAGCACCAGC-3' 5'-CTTGAACGACGACGACTTTGG-3'
CXCL15	5'-CCATGGGTGAAGGCTACTGT-3' 5'-TCTCAGGTCTCCCAAATGAAA-3'
HPRT	5'-CAAATCAAAAGTCTGGGGACGC-3' 5'-GCTTGCTGGTGAAAAGGACCTC-3'
MYH11	5'-AAGCTGCGGCTAGAGGTCA-3' 5'-CCCTCCCTTTGATGGCTGAG-3'
PPM1a	5'-GCGGAGCTGGACAAGTACC-3' 5'-GCCTTCCACCTGCTTCTTTA-3'
SMA	5'-GTCCCAGACATCAGGGAGTAA-3' 5'-TCGGATACTTCAGCGTCAGGA-3'
TNFα	5'-TAGCCAGGAGGGAGAACAGA-3' 5'-TTTTCTGGAGGGAGATGTGG-3'

Table 4: List of primer sequences for the real time RT - PCR



The PCR was performed on a stepOne Plus Real – Time PCR cycler (Applied Biosystems). HPRT was used as normalization gene. To exclude false – positive signals a melting curve was established. (Tab.5)

Step	Temperature	Time	Number of Cycles
Initiation	95°C	10 min	1
Denaturation	95°C	15 sec	40
Annealing	55°C	15 sec	
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

Table 5: Setup for quantitative real time RT - PCR

Gene expression analysis was done using the StepOne Software v2.1 (Applied Biosystems).

## **5.6 Tissue Sectioning**

### **5.6.1 *Paraffin Sections***

First, the harvested tissues were fixed in 4% paraformaldehyd over night. Following this the tissues were dehydrated and embedded in paraffin in small tissue blocks. The samples were then cut into 2,0 µm sections using a Leica microtome (Leica Microsystems, Vienna, Austria) and collected on SuperFrost Plus slides (Thermo Scientific, Braunschweig, Germany). Sections were either processed directly or stored at room temperature for later use.

### **5.6.2 *Cryosections***

The harvested tissues were directly frozen in isopentane / liquid nitrogen and frozen at -80°C until cutting. The frozen tissues were cut into 8,0 µm sections using a cryostat and dried at room temperature for 20 minutes or longer. Sections were either processed directly or stored at -80°C for later use.

## **5.7 Stainings**

### **5.7.1 *Slide Preparation***

#### **5.7.1.1 Deparaffinization**

Paraffin sections were first deparaffinized two times in the xylol substitute Roticlear (Carl Roth) for 20 minutes and then rehydrated in descending ethanol solutions (100%, 96%, 80%, 70%, 50%) for 30 seconds each. Then the slides were incubated in ddH<sub>2</sub>O for 5 minutes.

#### **5.7.1.2 Antigen Retrieval**

Antigen retrieval was only necessary for formerly paraffinized slides. To achieve this, the slides were put in Antigen Retrieval Solution (Dako, Glostrup, Denmark) and brought to the boil in a microwave. The slides were simmered for 20 minutes, cooled down to ambient afterwards and washed with 1 x PBS for 5 minutes.

#### **5.7.1.3 Fixation of Cryosections**

Cryosections were incubated in ice cold acetone for 3 minutes, quickly air dried and washed in 1 x PBS for 5 minutes.

### **5.7.2 Histological staining**

Paraffin sections were used for all histological stainings. Rehydrated slides were incubated in Hemalaun (1:10 dilution; Carl Roth) for 5 minutes and adjacently carefully washed in running tap water for 5 minutes. Afterwards the slides were dipped into 1% HCl in 70% ethanol two times and immediately washed in running tap water for 7 minutes. Then they were incubated in an Eosin solution (2% Eosin in 70% Ethanol with a few drops of acidic acid added; Carl Roth) for 2,5 minutes and dehydrated in ascending ethanol solutions (80%, 96%, 100%) for 30 seconds each. Finally, they were put into the xylene substitute for 1 more minute. After air drying the slides for 1 – 2 minutes they were mounted in Roti – mount (Carl Roth) and stored in the dark at room temperature.

### **5.7.3 Immunostaining**

Tissues were encircled with a DakoPen (Dako) and blocked in 5% goat serum in 1 x PBS for 1 hour. The slides were incubated in a wet chamber with the first antibody in an appropriate dilution in 5% goat serum in 1 x PBS at 4°C over night. Then the samples were washed in 1 x PBS for 5 minutes 3 times and incubated in a wet chamber with the adequate secondary antibody in an appropriate dilution in 1 x PBS at room temperature for 1 hour. (Tab.6) Subsequently the slides were washed in 1 x PBS for 5 minutes 3 times and mounted in PBS / glycerol (1:7). Slides and cover slip were sealed with nail polish.

If using biotinylated antibodies samples were additionally treated with an Avidin / Biotin blocking kit (Vector Laboratories, Burlingame, CA), prior to incubation with the primary antibody. Therefore the sections were incubated in Avidin for 15 minutes and washed once in 1 x PBS for 5 minutes. Afterwards they were incubated in Biotin for another 15 minutes and then washed in 3 times 1 x PBS for 5 minutes each.

If using fluorescent antibodies slides were protected from light by covering them with aluminum foil at all times. These slides were counterstained with Hoechst (stock: 1mg / ml; end conc.: 100ng / ml; diluted in 1 x PBS) for 5 minutes prior to the last washing step in 1 x PBS.

Slides incubated with antibodies linked to HRP were evaluated using DAB tablets (Sigma-Aldrich, Vienna, Austria). The enzymatic reaction was observed under a light microscope and stopped with ddH<sub>2</sub>O at an appropriate time point but no later than 7 minutes after induction. Then the slides were counterstained in Hemalaun for 5 minutes and washed in running tap water for another 5 minutes before they were sealed.

<b>Antibody / Antigen</b>	<b>Source (catalog # or equivalent)</b>	<b>Use</b>	<b>Dilution</b>
A555 goat anti rabbit	Invitrogen A-21428	IF-P, IF-F	1:2000
Biotin anti rabbit	Vector Laboratories BA-1000	IHC-P	1:400
Biotin anti goat	Vector Laboratories BA-9500	IHC-P	1:400
Biotin anti mouse	Vector Laboratories BA-9200	IHC-P	1:400
CD11b	eBioscience 13-0112	IF-F	1:200
F4/80	eBioscience 13-4801	IF-F	1:200
Gr-1	eBioscience 13-5931	IF-F	1:200
Ki67	Neomarkers RM-9106-S1	IF-P	1:200
Smooth muscle actin	Sigma C6198	IF-P, IF-F	1:400
Streptavidin, Alexa Fluor 555-conjugate	Invitrogen S32355	IF-F	1:500

Table 6: List of antibodies used for various stainings; Abbreviations: IF...Immunofluorescence, IHC...Immunohistochemistry, F...frozen sections, P...paraffin sections:

#### **5.7.4 Microscopy**

All microscopy work was done in collaboration with Dr. Andreas Birbach. Images were acquired on an Olympus AX470 microscope (Olympus, Vienna, Austria) equipped with F-View II (grayscale) and ColorViewII (color) digital cameras using the manufacturer's software (CellP; Olympus) and appropriate filter settings for fluorescence.

## **5.8 Statistical Analysis**

Statistical significance was determined by using one – way analysis of variance.

$P < 0,05$  was considered significant. Error bars in figures represent SEM.

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## 7 Discussion

As prostate cancer is one of the most frequently diagnosed cancers in males and is also one of the leading causes of cancer related deaths we wanted to shed some light upon the development of this disease. While certain aspects of the development of prostate cancer are thoroughly described the correlation of inflammation and prostate cancer is far from understood. Therefore we wanted to study the effects of a permanent inflammatory signaling on prostate cancer progression. To this end we analyzed changes in morphology and gene expression in mice featuring both a constitutively active inflammatory signaling and a heterozygous knock out of a tumor suppressor known to play an important role in prostate cancer (PTEN<sup>+/-</sup>-IKK2<sup>ca/ca</sup>) and compared them to wildtype and PTEN<sup>+/-</sup> mice.

Permanent NFκB signaling through the constitutive activation of the key mediator IKK2 alone did not lead to any striking changes in gene expression or morphology. Therefore we conclude that a constitutive active inflammatory signaling is not enough to transform the prostate tissue in this mouse model. The exact reasons for this are open for speculation but it is an interesting finding because IKK2 has been shown to act as an oncogenic kinase in the past and its activation on a PTEN<sup>+/-</sup> background produces a clear phenotype. One possible explanation could be that NFκB is only able to activate certain promoters in a proliferating epithelium but not in a quiescent epithelium such as the one found in the prostate. This hypothesis is backed up by data we obtained from studying mice that feature the same genetic alterations in keratinocytes of the skin instead of the prostate but show a clear phenotype. (Birbach et al. unpublished)

As already mentioned above, the activation of IKK2 on a PTEN<sup>+/-</sup> background leads to a drastic change in both morphology and gene expression. We could show that several cytokines, such as TNF $\alpha$  and chemokines, such as CXCL2, CXCL5, CXCL10 and CXCL15 are upregulated in PTEN<sup>+/-</sup>-IKK2<sup>ca/ca</sup> mice compared to wildtype and PTEN<sup>+/-</sup> mice.

Moreover, histological stainings with certain antibodies (F4/80, CD11b, Gr-1) clearly demonstrate that there is a strong influx of immune cells, such as granulocytes, macrophages and monocytes into the prostatic tissue. These immune cells seem to be attracted by the aforementioned chemokines of the epithelium and cytokine – activated stroma. This is supported by the fact that the stimulation of PE-PTEN<sup>+/-</sup>-IKK2<sup>ca/ca</sup> stromal cells with TNF $\alpha$  leads to an increased production of chemokines known to exert immune cell attracting functions.

Furthermore, we could show that the development of the severe hyperplasia we observed in the double transgenic mice is the result of increased proliferation and not apoptosis.

Another very interesting finding was that smooth muscle actin and myosin heavy chain 11 are drastically downregulated in PTEN<sup>+/-</sup>-IKK2<sup>ca/ca</sup> mice, which clearly leads to the loss of smooth muscle cells which is a known finding in prostate cancer. We could confirm this by histological stainings showing a fading amount of smooth muscle around the epithelium. Interestingly however, PTEN<sup>+/-</sup>-IKK2<sup>ca/ca</sup> mice did not develop an invasive phenotype at any time in their life. It is also important to note that the downregulation of smooth muscle markers is frequently associated with human prostate carcinoma. However, our results show that the loss of smooth muscle alone is not enough for a tumor to become invasive.

Inflammation and prostate cancer are undoubtedly interconnected on several levels. Although we did not observe the development of an invasive phenotype in our mouse model it might be very well possible that invasiveness occurs later in mouse life, succeeding additional mutations and alterations caused by persisting inflammatory signaling. Our results provide some evidence on how prostate cancer might be linked to inflammation. However, there is still a lot of work to be done to finally be able to understand this complex mechanism and come up with therapies or prophylaxes for the treatment of this unforgiving disease.

## 8 Appendage

### 8.1 Abbreviations

AID	Activation - Induced cytidine Deaminase
AP-1	Activator Protein 1
AR	Androgen Receptor
BPH	Benign Prostatic Hyperplasia
ca	constitutively active
cDNA	complementary DNA
Ck	Cytokeratin
Col	Collagen
COX	Cyclooxygenase
CRE	Cyclization Recombination
CXCL	CXC Ligand
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
EMT	Epithelial - Mesenchymal Transition
ER	Endoplasmic Reticulum
gDNA	genomic DNA
HCl	Hydrogen Chloride
HGPIN	High - Grade prostate Intraepithelial neoplasia
HIF	Hypoxia Inducible Factor
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
IF	Immunofluorescence
IHC	Immunohistochemistry
IKK	I $\kappa$ B Kinase
IL	Interleukin
I $\kappa$ B	Inhibitor of $\kappa$ B
MYC	myelocytomatosis oncogene
MYH11	Myosin Heavy Chain 11
NaCl	Sodium Chloride
NF $\kappa$ B	Nuclear Factor $\kappa$ B
PB	Probasin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIA	Proliferative Inflammatory Atrophy
PIN	Prostate Intraepithelial Neoplasia
PKB	Protein Kinase B
PPM1A	Protein Phosphatase 1A gene
pRB	Retinoblastoma Protein

PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
RAS	RAt Sarcoma oncogene
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
RT - PCR	Reverse Transcriptase - PCR
SEM	Standard Error of the Mean
SMA	Smooth Muscle Actin
SMAD	Mothers Against Decapentaplegic gene family
STAT	Signal Transducer and Activator of Transcription
TAM	Tumor Associated Macrophage
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor



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## 8.3 Curriculum Vitae

### Personal Data

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### Education

2004 – 2011	Diploma Study Microbiology / Genetics, University of Vienna, Vienna
2003 – 2004	Civilian Service, Geriatriezentrum St. Martin, Klosterneuburg
2002 – 2003	Diploma Study of Biotechnology, University of Natural Resources and Life Sciences, Vienna
1994 – 2002	Secondary School, BG/BRG Klosterneuburg, Klosterneuburg
1990 – 1994	Primary School, VS St. Andrä Wördern, St. Andrä

### Professional Career

2009 – 2010	Diplomathesis “Characteristics of prostate cells derived from an inflammatory model of prostate tumorigenesis”, Center for Physiology and Pharmacology, Medical University of Vienna, Vienna
2008	Practical Training “Fluorescence – in – situ – Hybridization (FISH), Department of Microbial Ecology, University of Vienna, Vienna
2008	Internship (2 months), Analytical Chemistry, Baxter BioScience, Vienna
2007	Internship (2 months), Analytical Chemistry, Baxter BioScience, Vienna

### Publications

2011	Birbach et al (2011). Persistent Inflammation Leads to Proliferative Neoplasia and Loss of Smooth Muscle Cells in a Prostate Tumor Model. Neoplasia, 11-524
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### Skills

Languages	German (first language) English (business fluent) French (basics)
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